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(54) Title: FLOW CYTOMETRY WITH NON-FLUORESCENT MICROBEADS

(57) Abstract

The present invention relates to antibody–coupled beads and to a method using the antibody–coupled beads for the detection of surface antigens. The method comprises the steps of contacting antibody–coupled beads with a cell sample under suitable condition for coupling of a specific antibody with surface antigen to forming a complex; and detecting the bead–antibody–antigen complex obtained by flow cytometry as an indication of density and size of the complex.

FLOW CYTOMETRY WITH NON-FLUORESCENT MICROBEADS

5   **BACKGROUND OF THE INVENTION**
    (a) _Field of the Invention_
        The invention relates to a method for detection
    of various surface antigens by flow cytometry, using
    non-fluorescent antibody-coupled microbeads.
10

    (b) _Description of Prior Art_
        Multiparameter flow cytometric analysis allows
    the characterization of several discrete cellular
    populations.   Routinely,   detection   of   up   to   four
15 surface antigens, following the staining of cells with
    a   cocktail   of   fluorochrome-conjugated   monoclonal
    antibodies (mAbs), is made possible using conventional
    cytometers   equipped   with   a   single-laser   emitting   at
    488 nm. Novel fluorochromes covering a wide range of
20 the   emission   spectra,   and   upgrades   to   commercial
    instruments have been reported. However, in spite of
    these improvements, efforts made to increase the number
    of simultaneously detectable surface markers have been
    hampered by spectral overlaps between fluorochromes,
25 and the elevated costs of additional laser beams. Yet,
    in order to get more accurate information about cell
    populations and differentiation stages, there is still
    a   definite   need   to   devise   methods   allowing   the
    assessment of extended numbers of surface proteins.
30   Microbeads   have   been   widely   used   for   various
    purposes in flow cytometry. Among them are the use of
    magnetic microbeads coated with specific mAbs for cell
    sorting. Beads coated with specific antigens have also
    been used as a solid phase support for the detection of
35 specific serum antibodies. Some studies report the use
    of antibody-coated beads to study cellular activation.

For example, fluorescent latex beads coated with specific monoclonal antibodies (mAbs) can be employed to crosslink cell surface receptor or to mimic opsonisation of particulate antigens and phagocytosis.

There exists a method for measuring surface antigen using optically distinct fine fluorochrome-coupled particles coated with antibodies. This method relies on the fluorescence detectors of a flow cytometer and thus limits the analysis to the number of such detectors available.

Furthermore, colloidal gold-conjugated antibodies have been used in combination with fluorochrome-conjugated antibodies for the detection of cellular markers by light microscopy. In an attempt to extend this technique to flow cytometric analysis, colloidal gold particles of 40 nm in diameter, coated with goat anti-mouse antibodies (GAM-G40), have been used to label lymphocytes. The first analysis of immunogold-stained mouse spleen cells was done using a two-laser flow cytometer, equipped with an argon ion laser providing wavelengths ranging from 452 to 529 nm, and a helium-neon laser providing 632 nm red light (Boehmer RM, King NJ, *J. Immunol. Meth.* 74:49-57, 1984). Cells stained with such gold particles were reported to appear more granular, and to be detectable through a shift in the side scatter (SSC) signal. Moreover, it was shown that optimal SSC signal amplification was achieved with the helium-neon laser emitting at 633 nm.

In U.S. Patent No. 5,248,772, Siiman et al. teach colloidal particles coated with a layer of aminodextran and a metallic layer such as gold, for instrumental analysis such as Raman spectroscopy or flow cytometry.

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In a more recent study, however, it was reported that detection of SSC amplification could be possible with an argon ion laser emitting at 488 nm (Festin R, et al., *J Immunol Meth* 101:23-28, 1987). Although this labeling protocol initially seemed promising and most appealing to flow cytometry users, it has never found its grounds in this discipline.

It would be highly desirable to be provided with a novel method of detection of surface antigens by flow cytometry using a non-fluorescent antibody-coupled microbeads.

It would be highly desirable to be provided with a method allowing for the detection of an additional cell surface marker without the need for an additional fluorochrome so that this marker could be used in flow cytometry without interfering with conventional fluorochromes used in such an assay.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a novel method of detection of surface antigens by flow cytometry using a non-fluorescent antibody-coupled microbeads.

Another aim of the present invention is to provide a method for the detection of an additional cell surface marker, which is independent of fluorescence detection and can be used without interference with conventional fluorochromes used in a flow cytometry assay.

In accordance with the present invention there is provided a method for the detection of surface antigens comprising the steps of:

a) contacting an antibody-coupled bead with a sample for a time sufficient for coupling the antibody-

coupled bead to bind at least one surface antigen to form a bead-antibody-antigen complex; and

b) detecting the complex obtained in a) by flow cytometry wherein the presence of the complex is indicative of the presence of the antigens.

Preferably the antibody-coupled bead recognizes at least one antigen.

The complex obtained produces light scattering in a flow cytometer, this light scattering is detected by a side scatter (SSC) signal as an indication of density of the complex.

The method of the present invention may further comprises, before the step a), the steps of contacting fluorochrome-coupled antibodies with the sample under suitable condition for coupling of the fluorochrome-coupled antibodies with other surface antigens.

The specific antibodies are preferably monoclonal antibodies.

Preferably, the antibodies-coupled beads are prepared by contacting beads with specific antibodies under suitable condition for coating these beads with the specific antibodies, thereby forming the antibodies-coupled beads.

The beads are preferably latex beads having a size of about 0.08 μm to 0.5 μm of diameter.

Also in accordance with the present invention, there is provided a non-fluorescent flow cytometric bead having antibody stably coupled thereto through an electrostatic attraction bond, wherein the antibody is retained on the bead under physiological condition.

Also in accordance with the present invention, there is provided a method for the detection of surface antigens comprising the steps of:

a) contacting a protein-coupled bead with a sample for a time sufficient for coupling the protein-

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coupled bead to bind at least one surface receptor to form a bead-protein-receptor complex; and

b) detecting the complex obtained in a) by flow cytometry wherein the presence of the complex is
5   indicative of the presence of the receptor.

The protein may be an antigen, a cytokine or any other ligand and the receptor may be an antibody, cytokine-receptor or any receptor of the ligand.

In the method of the present invention, the
10   protein-coupled bead preferably recognizes at least one antibody.

In a variant, the method of the present invention preferably further comprises before the step a) the step of contacting at least one fluorochrome-
15   coupled antibody with the sample under suitable condition for coupling of the at least one fluorochrome-coupled antibody with at least one additional surface antigen allowing for the detection of the at least one additional surface antigen.

20   In a further variant of the present invention, the method further comprises before the step a) the step of contacting at least one fluorochrome-coupled antigen with the sample under suitable condition for coupling of the at least one fluorochrome-coupled
25   antigen with at least one additional antibody allowing for the detection of the at least one additional antibody.

The protein-coupled bead are preferably prepared by contacting beads with a protein under
30   suitable condition for coating the beads with the protein, thereby forming the protein-coupled beads.

In another embodiment of the present invention, there is provided a non-fluorescent flow cytometric bead for detecting a specific antibody in a sample, the
35   non-fluorescent flow cytometric bead having at least

one protein stably coupled thereto through an electrostatic attraction bond, wherein the protein is retained on the bead under physiological condition and is bound by the specific antibody when present in the
5   sample.

In accordance with the method of the present invention there is provided a novel use of microbeads in flow cytometry. More particularly, there is provided the use of a non-fluorescent flow cytometric
10   bead as defined above in flow cytometry assay for the detection of an additional cell surface marker which is independent of fluorescence detection, the bead used in flow cytometry assay not interfering with conventional fluorochromes used in the flow cytometry assay.

15   The method of the present invention is very useful for upgrading the surface antigen detection capability of any flow cytometry units, since it can easily be used in combination with conventional fluorochrome-conjugated mAbs-based staining. For
20   example, in accordance with the method of the present invention, it is possible to detect five distinct surface markers, using a single 488nm-emitting laser cytometer, equipped with four fluorescence detectors. Furthermore, the method of the present invention allows
25   for the detection of microbeads-antibody-antigen complex without the need for electronic compensation, unlike conventional methods.

BRIEF DESCRIPTION OF THE DRAWINGS

30   Fig. 1 represents graphics illustrating linear forward scattering (FSC) versus log side scattering (SSC log) distributions (left panels), and SSC log histogram profiles (right panels) for cells incubated with either non-coated (dashed lines) or anti-CD4 mAb-

coated beads (dark lines) of 0.1 μm, at varying bead to cell ratios;

Fig. 2 represents log side scattering (SSC log) histograms, for cells incubated with control (dashed lines) or anti-CD4 mAb-coated beads (dark lines) of 0.08 μm, 0.1 μm or 0.5 μm;

Fig. 3A represents linear forward scattering (FSC) versus log side scattering (SSC log) contour plots, and SSC log histograms, for cells pre-incubated in the presence (two right panels) or the absence (two left panels) or of soluble anti-CD4 mAb, followed by anti-CD4 mAb-coated beads (0.1 μm, 1.6 x 10^5 beads/cell);

Fig. 3B represents FSC versus SSC log profile and FL-2 histogram (two left panels) for cells incubated in the presence of both anti-CD4 (clone GK1.5) mAb-coated beads (0.1 μm, 1.6 x 10^5 beads : cell) and PE-conjugated anti-CD4 (clone H129.19) mAbs compared to FSC versus SSC log contour plot restricted to the FL-2-positive or the FL-2-negative populations as illustrates in the two right panels;

Fig. 4 illustrates an immunofluorescence micrograph of the bead-cell complexes, representing lymph node cells first incubated with Hoechst 33342 (stains all the cells in blue), and then with anti-CD4 mAb-coated beads (0.1 μm, 1.6 x 10^5 beads : cell), which exceptionally for the purpose of this Fig. 4 was conjugated to a green fluorochrome;

Fig. 5 represents log side scatter (SSC log) and FL-1 to FL-4 log histograms for cells stained with control (A) or anti-CD4 mAb-coated beads (B), and with FITC-conjugated anti-CD45RB (Fl1), PE-conjugated anti-CD45R (Fl2), RED613™-conjugated anti-CD8 (Fl3) and biotin-conjugated anti-TCRβ, revealed with RED670™-conjugated streptavidin (Fl4); and

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Fig. 6 represents a SSC log versus FL3 log contour plot (typical CD4/CD8 profile), ungated or gated from CD45R+, TCRβ+, CD45RB-, CD45RB^{low} and CD45RB^{high} for cells stained with anti-CD4 mAb-coated

5   beads and FITC-, PE-, RED613™-, and RED670™-conjugated mAbs, as described in Fig. 5.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with a preferred embodiment of

10 the present invention, there is provided an alternative reliable method, based on the staining of cells with non-fluorescent 0.1 μm latex microbeads coated with mAbs directed against membrane proteins. Cells expressing a given surface antigen are readily detected

15 since the binding of mAb-coated beads caused a dramatic shift in the logarithmic SSC profile. The specificity of the present method was confirmed by the demonstration that an identical proportion of cells was stained following the labeling with either

20 fluorochrome-conjugated mAbs or mAbs-coated beads.

Moreover, the method of the present invention is successfully used in combination with conventional staining with fluorochrome-conjugated mAbs. A single 488 nm emitting laser flow cytometer is used to perform

25 the simultaneous detection of five surface markers, with mAb-coated beads according with the present invention, and fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, RED613™-, or RED670™-conjugated mAbs.

30 The method of the present invention is fast, inexpensive, and does not interfere with conventional detection of fluorochrome-conjugated markers. Indeed, mAb-coated latex beads can be used as an additional label, thereby allowing to upgrade multiparametric flow

35 cytometry analysis.

Microbeads

Microbeads which are used in accordance with the present invention may be made of a polymer such as polystyrene, latex, etc.

Sulfate polystyrene latex microbeads used in accordance with the present invention were obtained from Interfacial Dynamics Corporation (Portland, OR). The mean diameters of the beads (as determined from transmission electron microscopy), the percentage solid, the particle number per ml, the specific surface area, the density and the refractive index of polystyrene are given for each bead preparations in Table 1.

Table 1
Physical specifications for microbeads

mean diameter* (μm) (coefficient of variation)	0.53 0.027 (CV 5.1%)	±	0.098 0.005 (CV 5.4%)	±	0.079 0.009 (CV 11.2%)	±
per cent solids (g/100 ml)	8.2 0.1	±	8.2 0.1	±	8.2 0.1	±
particle number per ml	1.0×10^{12}		1.6×10^{14}		3.0×10^{14}	
specific surface area (cm^2/g)	1.1×10^{5}		5.8×10^{5}		7.2×10^{5}	
density of polystyrene (20°C, g/cm^3)	1.055		1.055		1.055	
refractive index (590 nm, 20°C)	1.591		1.591		1.591	
catalog number	1-500		1-100		1-80	

* Referred in the text as 0.5 μm, 0.1 μm and 0.08 μm.

Monoclonal antibodies

Monoclonal antibodies (mAbs) used in accordance with the present invention were anti-CD4 (clone GK1.5, ATCC TIB207), FITC-conjugated anti-CD45RB (clone MB23G2, ATCC HB220), PE-conjugated anti-CD45R (clone RA3-6B2; PharMingen, SanDiego, CA), biotin-conjugated anti-TCRβ (clone H57-597, ATCC HB218), PE-conjugated anti-CD4 (clone H129.19; GIBCO BRL, Grand Island, NY), RED613™-conjugated anti-CD8 alpha-chain (clone 53-6.72; GIBCO BRL), RED670™-conjugated streptavidin (GIBCO BRL), FITC-conjugated anti-rat Ig k-chain (MAR-18.5, ATCC TIB216), and DDAF (FITC-conjugated sheep anti-mouse Ig, F(ab')2 fragments; Silenus Laboratories, Hawthorn, Australia). Purified as well as FITC- and biotin-conjugated mAbs were prepared in the laboratory using standard protocols. All antibodies were titrated to ensure their usage at saturating concentrations.

Antibody coating on microbeads

Latex microbeads adsorb proteins strongly and practically irreversibly via electrostatic interactions at physiological condition. The physiological condition is defined as being the condition under which the protein is still in a native form or functional. Beads of 0.5, 0.1 and 0.08 μm in diameter were used. The surface (in μm^2) of these beads was calculated (0.900, 0.030 and 0.020 μm^2, respectively), and the number of beads to be coated with a given mAb concentration was adjusted in order to obtain a similar total surface. This ensured that equivalent densities of antibodies were obtained on each set of beads. Therefore, the amount of beads of each size required to reach a total surface of 4.8 x 10^{10} μm^2 was calculated. Microbeads coating was performed in 1.5 ml Eppendorf tubes, in a total volume of 1 ml of purified anti-CD4 antibody (clone GK1,5), at the saturating concentration

of 200 μg/ml in phosphate buffered saline solution (PBS). As a control, the same amount of beads were incubated with PBS only. Bead suspensions were mixed by end-to-end rotation, overnight at 4°C. The beads

5 were then centrifuged (15,000g, 5 minutes) and the supernatant was removed. Blocking of the excess hydrophobic binding sites on particle's surface was achieved by incubating the beads in 0.5 ml of whole calf serum (30 min. at 37°C). Finally, the beads were

10 washed twice in PBS with 2% calf serum and 1% azide (PBS/CS/AZ), resuspended in a final volume of 1 ml of PBS/CS/AZ and vortexed vigorously to obtain homogenous suspension (a fine pipette was used to break the aggregates when necessary). Routinely, an aliquot of

15 mAb coated beads were stained with DDAF, to ensure that all beads were homogeneously coated with anti-CD4 mAb on their surface. An ELISA was performed to determine the saturating concentration of anti-CD4 mAb for each bead preparation. Large amounts of beads have been

20 coated with mAbs and kept at 4°C, and were shown to be stable for at least two weeks.

Cell staining with fluorochrome-conjugated antibodies and mAb-coated beads

Lymph nodes were removed from 1-2 months old

25 mice. A cell suspension was prepared in PBS/CS/AZ, at a final concentration of 4 x 10^6 cells/ml. The cells were distributed in a conical wells plate (100 μl of the cell suspension, 4 x 10^5 cells per well), centrifuged at 100g for five minutes, and the

30 supernatants were removed.

Lymphocytes were stained as described hereinafter. Briefly, 4 x 10^5 cells were first stained in 50 μl of PBS/CS/AZ, containing "blocking solution" (5 μg/ml of human γ-globulins, Sigma, St-Louis, MO),

35 and culture supernatant (clone 2.4G2) containing a rat

anti-mouse FcγIII receptor mAb) with a mixture of FITC-conjugated anti-CD45RB, PE-conjugated anti-CD45R, RED613™-conjugated anti-CD8 and biotin-conjugated anti-TCRβ antibodies. The cells were incubated for 30 min. at 4°C. After washing the cells twice in PBS/CS/AZ, the pellets were resuspended in 50 μl of RED670™-conjugated streptavidin in PBS/CS/AZ and incubated for 30 min. at 4°C. In a third step, anti-CD4 mAb-coated beads (the optimal bead : cell ratio was determined for each set of bead size; see Example 1 and Fig. 1) were added to the stained cells, and the volume was adjusted to 60 μl. The suspensions containing cells and beads were spun at 100g for 5 min. at 4°C (it was found to be preferable to centrifuge/resuspend cells with beads two times in order to obtain the highest scatter signal on the flow cytometer). The incubation of cells with beads was continued for an additional 30 min. at 4°C. The cells were then washed twice and resuspended in a final volume of 1 ml of PBS/CS/AZ, in polypropylene microtubes (National Scientific Supply Co, Claremont, CA).

Flow cytometry

Samples were run on a Coulter XL™ flow cytometer (Coulter Corporation, Hialeah, FL) equipped with a 15 mW 488 nm argon laser with 525, 575, 610 and 675 nm detectors, and analyzed using the Coulter System II software. Linear forward light scatter (FSC), logarithmic SSC and fluorescences were recorded at low flow rate (200 events/second). A total of 25,000 or 50,000 live cells were acquired for each sample.

Fluorescence microscopy

For microscopic assessment of bead binding to cells, 1×10^6 lymphocytes were first incubated (30 minutes at 37°C) with Hoechst 33342 (Sigma), at a final concentration of 50 μg/ml, in 1 ml of RPMI-1640 (GIBCO

BRL) with 10% fetal calf serum. The cells were washed
twice, and were then stained with anti-CD4 mAb-coated
beads of 0.1 μm, at a bead to cell ratio of 1.6 x 10^5
beads per cell (determined to be optimal). Finally,
5   FITC-conjugated MAR-18.5 (which can recognize anti-CD4
mAb) was used to label the beads. The stained cells
were concentrated (2 x 10^6 cells in 5 μl RPMI) and used
immediately.   Samples   were   observed   (400X
amplification)   on   an   epi-fluorescent   Leitz   DM-RB
10 microscope (Leica, Saint-Laurent, CANADA) equipped with
a Xe-Hg lamp and filters A and I3, for Hoechst and FITC
detection, respectively.

The novel method of the present invention can
thus be used to circumvent limitations imposed by the
15 prior art.   Although   various   beads   having   electric
charges could be used in accordance with the present
invention, latex beads are preferred.   Furthermore,
various sizes of beads or latex beads could also be
used in the method of the instant invention. However,
20 0.1 μm beads are preferred. It is also shown in the
following examples that similar proportions of cells
were   stained   either   with   mAb-coated   beads   or
fluorochrome-conjugated mAbs, therefore attesting the
specificity of the method of the present invention.
25 Importantly, it is also demonstrated that the novel
staining method of the present invention could be
combined   to   conventional   staining   protocols   with
fluorochrome conjugated mAbs, thereby providing new
means to increase the number of detectable surface
30 markers on standard flow cytometers.

In this study, an example of multiparametric
analysis is provided, using both mAb-coated beads and
fluorochrome-conjugated mAbs, to stain murine lymph
node cells.   From such experiments, the following
35 conclusions regarding the use of this labeling protocol

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can be drawn. The cells to be stained with mAb-coated
beads should have a relatively homogeneous SSC profile,
as detection is based on SSC shift. For similar
reasons, it is preferred in accordance with one
5   embodiment of the invention that the mAbs used to coat
the beads should be directed against an abundant
surface molecule, found on a large proportion of cells.
Moreover, when performing multi-step staining procedure
with both mAb-coated beads and fluorochrome-conjugated
10 mAbs, mAb-coated beads is preferably be added last,
since multiple washing steps may cause dissociation of
bead-cell complexes. It is worth noting that because
of the steric hindrance of the mAb-coated beads, it was
impossible to occupy all the available epitopes at the
15 surface of the cell. This was demonstrated when cells
were successively stained with mAb-coated beads,
followed by fluorochrome-conjugated mAb recognizing the
same epitope. In this case, the fluorescent mAb could
easily find its epitope on the bead-stained cells,
20 since many binding sites were still free. Steric
hindrance may thus provide an explanation for the fact
that the side scatter shift cannot be increased over a
given bead : cell ratio (see Fig. 1). The bead to cell
ratios (b:c) used in Fig. 1 were 2×10^4, 4×10^4,
25 8×10^4, 1.6×10^5 and 2.4×10^5 beads per cell. A
total of 25,000 live cells were cumulated for each
sample. The SSC log histograms are gated from FSC/SSC
log contour plot to exclude dead cells. Percentages of
SSC^{hi} cells and mean fluorescence intensities (MFI) are
30 shown.   These results are representative of three
experiments.

        The present invention will be more readily un-
derstood by referring to the following examples which
are given to illustrate the invention rather than to
35 limit its scope.

EXAMPLE I
Detection of CD4 on cells stained with mAb-coated beads and assessment of optimal bead : cell ratio

5

A first experiment was carried out to "titrate" the amount of beads required to achieve the optimal amplification of the side scatter signal. Murine lymph nodes cells were incubated with increasing numbers of
10   non-coated beads (control) or anti-CD4 mAb-coated, 0.1 μm beads. The bead to cell ratios used were 2×10^4, 4×10^4, 8×10^4, 1.6×10^5 and 2.4×10^5 beads per cell. Samples were analyzed on a Coulter XL™ flow cytometer. Fig. 1 shows linear forward scatter signal
15   (FSC) versus side scatter signal (SSC) log distributions (left panels), and SSC log histograms profiles (right panels) for cells incubated with either non-coated (dashed lines) or anti-CD4 mAb-coated beads (dark lines). The SSC log histograms are gated from
20   FSC/SSC log contour plot to exclude dead cells.

A shift in the side scatter profile was readily detected for lymph node cells stained with anti-CD4 mAb-coated beads, but not with control beads. It can be seen from Fig. 1 that the SSC signal is dramatically
25   increased, and the dead cells (low FSC) are not stained. The signal amplification due to the bead label was sufficient for quantitative discrimination between positive and negative cells. Optimal staining, allowing the best discrimination between CD4- and CD4+
30   cells as reflected by a modulation in the SSC profile, was obtained with a 1.6×10^5 bead : cell ratio (Fig. 1). This labeling condition was "saturating", as adding more beads (2.4×10^5 beads : cell) did not result in an larger proportion of positive cells, nor
35   in a greater SSC shift (see the mean fluorescence intensity). Thus, all following experiments performed

with 0.1 μm beads were done using this ratio. CD4
detection with microbeads is used as a prototype, but
staining with other antibodies (e.g. anti-CD3 mAbs-
coated beads) have also been tested and has shown to
5 work properly.

EXAMPLE II
Cell labeling with beads of various sizes

10   Other size of beads were then investigated as
to whether the beads would allow a better amplification
of the SSC profile. Bead suspensions of three
different sizes (0.08, 0.1 and 0.5 μm in diameter) were
tested for their ability to induce a SSC shift. Lymph
15 nodes cells were labeled with control or anti-CD4 mAb-
coated beads of 0.08, 0.1 or 0.5 μm, each used at the
bead : cell ratio found to be optimal (1.6×10^5 bead
per cell for 0.08 and 0.1 μm, and 2.4×10^3 bead per
cell for 0.5 μm). SSC log histograms of cells
20 incubated with control (dashed lines) or anti-CD4 mAb-
coated beads (dark lines) are displayed in Fig. 2.
Each bead preparation was used at the bead : cell ratio
found to be optimal (1.6×10^5 beads : cell for 0.08 μm
and for 0.1 μm, and 2.4×10^3 beads : cell for 0.5 μm).
25 A total of 25,000 live cells were cumulated for each
sample. The SSC log histograms are gated from FSC/SSC
log dot plot to exclude dead cells. Percentages of
SSC^{hi} cells, mean fluorescence intensities (MFI) and
coefficient of variation (CV) are shown.
30   SSC shift was greater as the size of the beads
was increased (see MFI). However, non-specific binding
occurred with the control (non-coated) beads of 0.5 μm.
Moreover, the SSC^{high} population obtained with the anti-
CD4 mAb-coated beads of 0.5 μm was not homogenous
35 (large CV). It was routinely found that 0.1 μm beads

provided the best resolution between CD4- and CD4+ cells, without causing non-specific binding. Therefore, this size of beads was used in all the following examples.

EXAMPLE III
Specificity of the labeling technique
with mAb-coated beads

To assess the specificity of the mAb-coated beads staining protocol, the ability of soluble purified anti-CD4 mAb (GK1.5) to block the staining of lymphocytes by anti-CD4 mAb (GK1.5)-coated beads was evaluated. Lymph nodes cells were incubated in the absence or in the presence of soluble GK1.5, and then with optimal amounts of anti-CD4 mAb-coated beads (0.1 μm, 1.6 x 10^5 beads/cell). FSC versus SSC log contour plots, and gated SSC log histograms, are shown in Fig. 3A. Cell labeling with anti-CD4 mAb-coated beads was totally abrogated by the prior incubation of cells with soluble purified anti-CD4 mAb.

In Fig. 3A, a staining with anti-CD4 mAb-coated beads is blocked by prior incubation of the cells with purified soluble anti-CD4 antibody. The SSC log histograms are gated from FSC/SSC log contour plot to exclude dead cells.

The proportion of lymph node cells labeled with mAb-coated beads were then compared to that obtained with conventional fluorochrome-conjugated mAbs. For this purpose, the cells were successively incubated with anti-CD4 (GK1.5) mAb-coated beads (0.1 μm, 1.6 x 10^5 beads : cell) followed by PE-conjugated anti-CD4 (H129.19) mAbs. These two mAbs recognized distinct epitopes on the CD4 molecule. FSC versus SSC log profile (showing the anti-CD4 mAb-coated beads staining), as well as FL-2 histogram (showing the PE-

conjugated anti-CD4 staining), are illustrated in Fig. 3B. When the FSC versus SSC log contour plot was restricted to the PE-positive cells, the analysis clearly showed that all these cells were SSC^{high}, that is, all the CD4+ cells were successfully revealed by the staining with mAb-coated beads. The reciprocal analysis indicated that all PE-negative cells were SSC^{low}. Moreover, staining with either anti-CD4 mAb-coated beads or PE-conjugated anti-CD4 yielded populations of identical sizes (33% of total lymph nodes lymphocytes are CD4+), indicating good accuracy of the former technique.

In Fig. 3B, the proportion of lymph node cells labeled with mAb-coated beads is identical to that obtained with fluorochrome-conjugated mAbs. The FL-2 log histograms are gated from FSC/SSC log contour plot to exclude dead cells. FSC versus SSC log contour plot, restricted to the FL-2-positive or the FL-2-negative populations, are also illustrated (two right panels). A total of 25,000 live cells were cumulated for each sample. Three independent experiments showed similar results.

The absence of doublets of bead-stained cells was confirmed in a cell mixture experiment. Lymphocytes stained with FITC-conjugated anti-CD3 (clone 145-2C11) were mixed equally (1:1) with lymphocytes stained with biotin-conjugated anti-TCR (clone H57-597) revealed by RED670™-conjugated streptavidin, and then stained with anti-CD4 coated beads. No doublets were present since the gating on the CD4+ population yielded exclusively FITC+ or RED670+ cells. Taken together, these results indicated that staining with mAb-coated beads represents a reliable alternative to the conventional staining of cells with fluorochrome-conjugated mAbs.

In Fig. 4, a photomicrography of lymph nodes cells stained with anti-CD4 mAb-coated beads is depicted. The cells were stained with Hoechst 33342, a vital dye that stains all cells in blue, and with anti-
5   CD4 mAb-coated beads, as described above. Anti-CD4 mAbs (GK1.5) bound to the beads were revealed by the addition of FITC-conjugated anti-rat Ig k-chain mAbs (MAR18.5). This allowed to visualize the CD4+ (FITC+, green) cells among all lymphocytes (Hoechst+, blue)
10 (double exposure of FITC and Hoechst, 400X amplification).

EXAMPLE IV
Detection of multiple surface markers by
15 combining mAb-coated bead staining and
labeling with fluorochrome-conjugated mAbs

The data presented so far relate the specificity of the mAb-coated bead staining protocol
20 and provide a basis for setting-up multi-parameter labeling experiments. Lymph node cells were stained with mAbs directed against five distinct surface antigens, using anti-CD4 mAb-coated beads, FITC-conjugated anti-CD45RB, PE-conjugated anti-CD45R,
25 RED613™-conjugated anti-CD8 and biotin-conjugated anti-TCRβ, revealed with RED670™-conjugated streptavidin. These markers were chosen because they are commonly used to study distinct subsets within the T-cell and the B-cell compartments of lymph node cells.
30 Hence, T lymphocytes are subdivided in two populations, characterized by the exclusive expression of the CD4 marker on helper T lymphocytes, and the CD8 marker on cytotoxic T lymphocytes. Naive and memory phenotype in T cells can be defined by the level of expression of
35 CD45RB, and the CD45R marker is typically found on B lymphocytes.

Flow cytometric analyses were performed, following the gating of cells based on the FSC versus SSC log dot plot, to generate histograms for SSC, FL-1, FL-2, FL-3 and FL-4 log profiles (Fig. 5A with control beads and Fig. 5B with anti-CD4 mAb-coated beads). Proportions of positive cells for each markers were found to match those described in the literature. No interference was seen with the simultaneous use of mAb-coated beads and fluorochrome-conjugated mAbs.

Again, cells labeled with anti-CD4 mAb-coated beads produced a distinct and well-defined population (SSC^{high}) which could be gated upon. Characterization of the CD4+ subset by electronic gating could be achieved by tracing the FL-1, FL2, FL3, and FL-4 log histograms (Fig. 5C). Such analysis clearly pointed out that cells contained within the SSC^{high} subset represent an homogenous population, expressing markers known to be present on CD4+ T-lymphocytes. Indeed, the SSC^{high} (CD4+) cells were found to be $CD45RB^{lo}$, CD45R-, CD8- and TCRβ+. In contrast, SSC^{low} cells represented a mixed population, which was composed of CD45RB- and $CD45RB^{high}$ cells, CD45R+ B lymphocytes and CD8+ T lymphocytes (Fig. 5D). Similar results were obtained when cells were analyzed by conventional four-color immunofluorescence (using PE-conjugated anti-CD4 mAbs instead of anti-CD4 mAb-coated beads).

An analysis of combined mAb coated-bead staining and fluorochrome-conjugated mAb labeling, by gating on bead-stained cell population is illustrated in Fig. 5. All these histograms are gated from FSC/SSC log dot plot to exclude dead cells. FL-1 to FL-4 log histograms, restricted to the SSC^{high} population (CD4+) (C) or the SSC^{low} population (CD4-) (D), are also illustrated. A total of 50,000 live cells were cumulated. Note that most of the FITC-, PE-, R613™-

and R670™-negative cells are in the Y-axis, due to the
Coulter system. One representative experiment, that
has been repeated three times, is shown.

  A reciprocal analysis was carried out to
5   further exemplify the use of the method of the present
invention in subset characterization. Hence, a typical
CD4 (SSC log) versus CD8 (FL3 log) contour plot profile
is depicted, for cells restricted to the following
populations: CD45R+, TCRβ+, CD45RB-, CD45RB^{lo}, and
10 CD45RB^{high} (Fig. 6). Although the CD45RB- population
contained a small proportion of CD4+ cells, it was
mainly constituted by CD4- and CD8-, thus representing
the B-cell compartment. On the other hand, the
CD45RB^{lo} and CD45RB^{high} subsets were mostly CD4+ and
15 CD8+, respectively. As expected, this analysis also
revealed that CD45R+ cells (B lymphocytes) express
neither CD4 or CD8 markers, and that TCRβ+ lymphocytes
contained exclusively CD4 or CD8 expressing cells.
Similar results were obtained when lymph node cells
20 were analyzed by conventional four-color
immunofluorescence, to determine the relative
proportions of CD4+ and CD8+ populations within these
different subsets.

  Fig. 6 shows the detection of CD4 and CD8
25 expression within distinct CD45RB subsets, using mAb
coated bead labeling in combination with fluorochrome-
conjugated mAbs. A total of 50,000 live cells were
cumulated. Note that most of the R613™-negative cells
are in the Y-axis, due the Coulter system. These
30 results are representative of three independent
experiments.

  While the invention has been described in con-
nection with specific embodiments thereof, it will be
understood that it is capable of further modifications
35 and this application is intended to cover any varia-

tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1.   A method for the detection of surface antigens comprising the steps of:

        a) contacting an antibody-coupled bead with a sample for a time sufficient for coupling said antibody-coupled bead to bind at least one surface antigen to form a bead-antibody-antigen complex; and

        b) detecting the complex obtained in a) by flow cytometry wherein the presence of said complex is indicative of the presence of said antigen.

2.   The method of claim 1, wherein said antibody-coupled bead is a non-fluorescent antibody-coupled bead.

3.   The method of claim 1 or 2, wherein said antibody-coupled bead recognizes at least one antigen.

4.   The method of claim 1, 2 or 3, wherein said complex produces light scattering in a flow cytometer, said light scattering being detected by a side scatter (SSC) signal as an indication of density of the complex.

5.   The method of claim 1, 2, 3 or 4, further comprising before the step a) the step of contacting at least one fluorochrome-coupled antibody with the sample under suitable condition for coupling of said at least one fluorochrome-coupled antibody with at least one additional surface antigen allowing for the detection of said at least one additional surface antigen.

6.   The method of claim 1, 2, 3 or 4, further comprising before the step a) the step of contacting at least one fluorochrome-coupled antigen with the sample under suitable condition for coupling of said at least one fluorochrome-coupled antigen with at least one additional antibody allowing for the detection of said at least one additional antibody.

7.   The method of claim 1, 2, 3, 4, 5 or 6, wherein the antibody-coupled bead is prepared by contacting beads with a specific antibody under suitable condition for coating said beads with said specific antibody, thereby forming said antibody-coupled beads.

8.   The method of claim 7, wherein the specific antibody is a monoclonal antibody.

9.   The method of claim 7, wherein the specific antibody is a polyclonal antibody.

10.   The method of claim 6, 7, 8 or 9, wherein the bead has a size of about 0.08 μm to 0.5 μm of diameter.

11.   The method of claim 6, 7, 8, 9 or 10, wherein the bead is a latex bead.

12.   A non-fluorescent flow cytometric bead having at least one antibody stably coupled thereto through an electrostatic attraction bond, wherein said antibody is retained on said bead under physiological condition.

13.   The non-fluorescent flow cytometric bead of
claim 12, wherein the bead has a size of about 0.08
μm to 0.5 μm of diameter.

14.   The non-fluorescent flow cytometric bead of
claim 12 or 13, wherein the bead is a latex bead.

15.   A method for the detection of surface
antigens comprising the steps of:
       a) contacting a protein-coupled bead with a
sample for a time sufficient for coupling said
protein-coupled bead to bind at least one surface
receptor to form a bead-protein-receptor complex;
and
       b) detecting the complex obtained in a) by
flow cytometry wherein the presence of said complex
is indicative of the presence of said receptor.

16.   The method of claim 15, wherein said protein
is an antigen and said receptor is an antibody.

17.   The method of claim 15 or 16, wherein said
protein-coupled bead is a non-fluorescent protein-
coupled bead.

18.   The method of claim 16, wherein said protein-
coupled bead recognizes at least one antibody.

19.   The method of claim 15, 16, 17 or 18, wherein
said complex produces light scattering in a flow
cytometer, said light scattering being detected by a
side scatter (SSC) signal as an indication of
density of the complex.

20.   The method of claim 15, 16, 17, 18 or 19,
further comprising before the step a) the step of
contacting   at   least   one   fluorochrome-coupled
antibody with the sample under suitable condition
for coupling of said at least one fluorochrome-
coupled antibody with at least one additional
surface antigen allowing for the detection of said
at least one additional surface antigen.

21.   The method of claim 15, 16, 17, 18 or 19,
further comprising before the step a) the step of
contacting at least one fluorochrome-coupled antigen
with the sample under suitable condition for
coupling of said at least one fluorochrome-coupled
antigen with at least one additional antibody
allowing for the detection of said at least one
additional antibody.

22.   The method of claim 15, 16, 17, 18, 19, 20 or
21, wherein the protein-coupled bead is prepared by
contacting beads with a protein under suitable
condition for coating said beads with said protein,
thereby forming said protein-coupled beads.

23.   The method of claim 22, wherein the bead has
a size of about 0.08 μm to 0.5 μm of diameter.

24.   The method of claim 22 or 23, wherein the
bead is a latex bead.

25.   A non-fluorescent flow cytometric bead for
detecting a specific antibody in a sample, said non-
fluorescent flow cytometric bead having at least one
protein stably coupled thereto through an
electrostatic attraction bond, wherein said protein

is retained on said bead under physiological condition and is bound by said specific antibody when present in said sample.

26.   The non-fluorescent flow cytometric bead of claim 25, wherein the bead has a size of about 0.08 μm to 0.5 μm of diameter.

27.   The non-fluorescent flow cytometric bead of claim 25 or 26, wherein the bead is a latex bead.

28.   Use of a non-fluorescent flow cytometric bead as defined in claim 12, 13, 14, 25, 26 or 27 in flow cytometry assay for the detection of an additional cell surface marker which is independent of fluorescence detection, said bead used in flow cytometry assay not interfering with conventional fluorochromes used in said flow cytometry assay.

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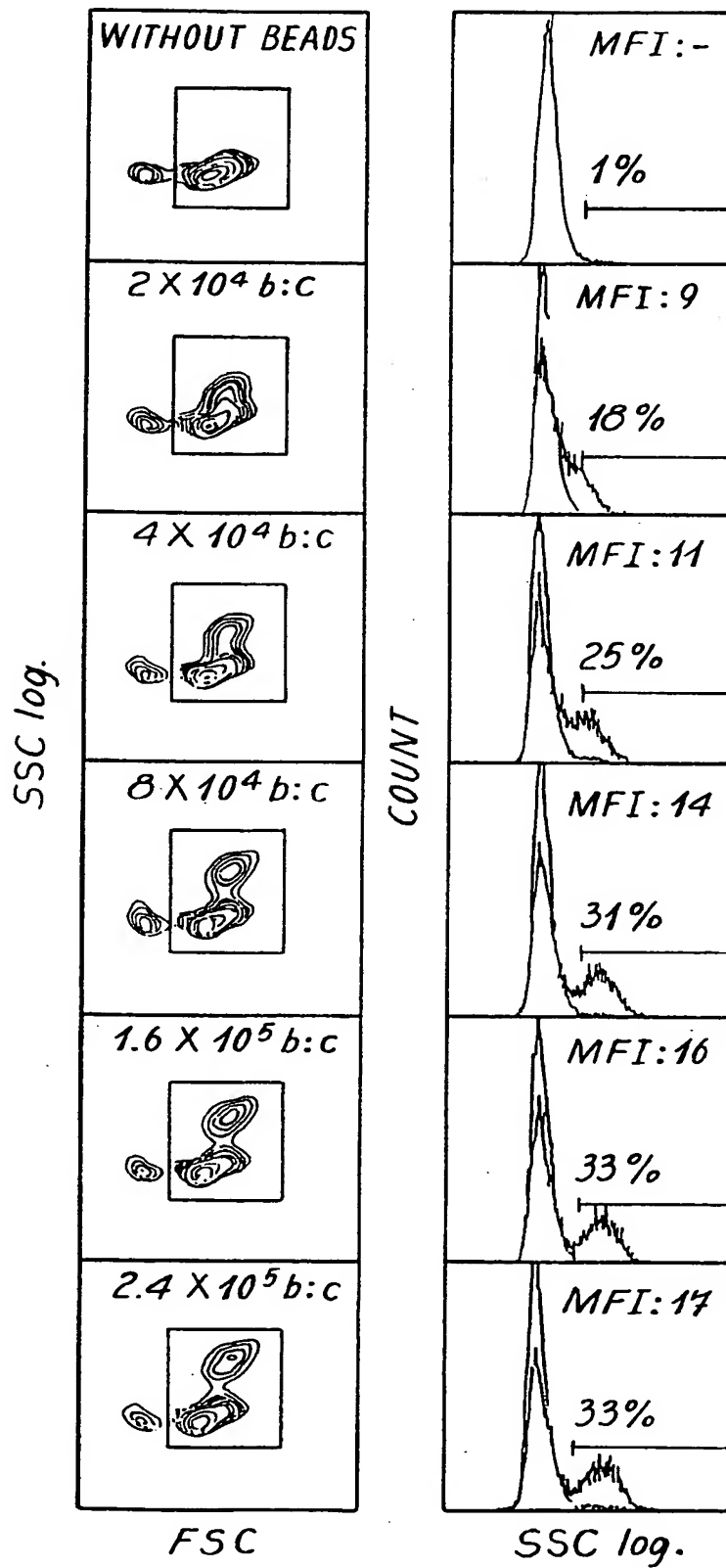
Fig. 1

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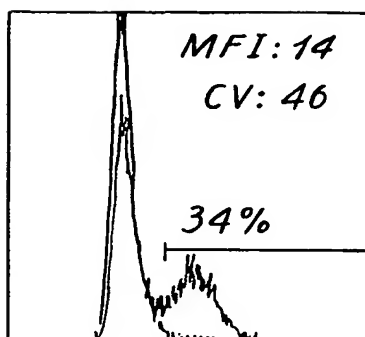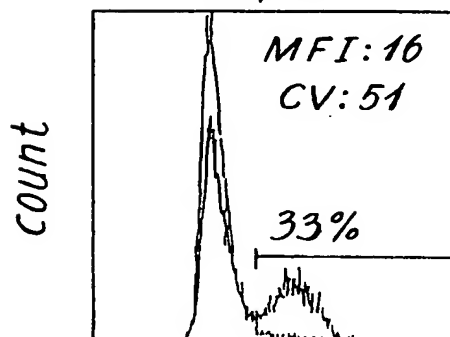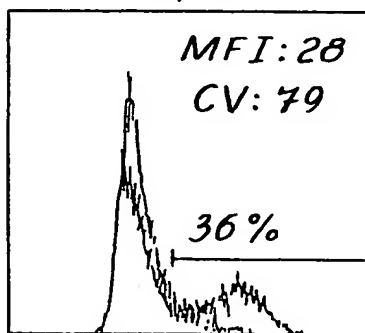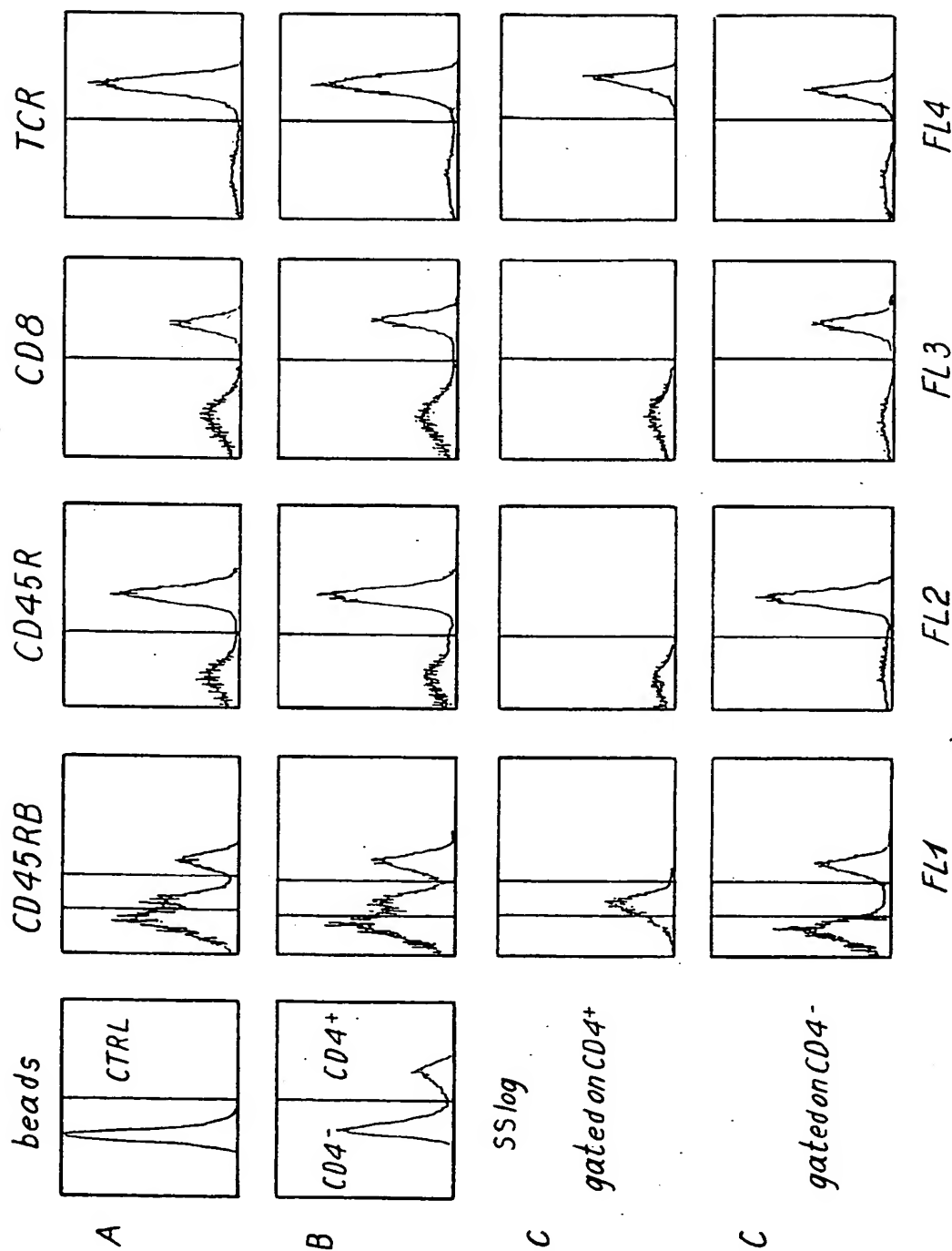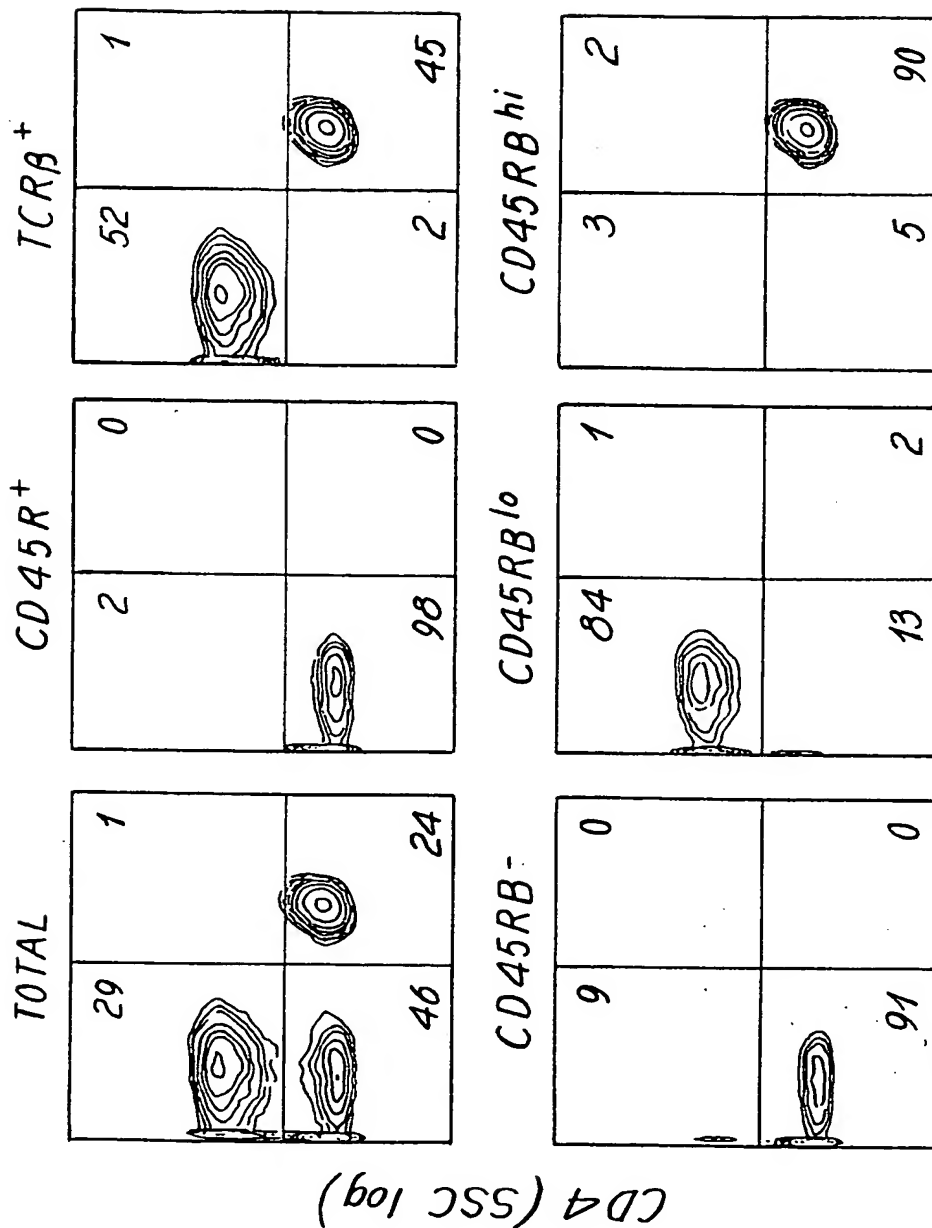
Fig. 2

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Fig. 3A

Fig. 3B

Fig. 4

Fig. 5

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fig. 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   G01N33/58   G01N33/543   G01N33/577   G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 552 086 A (SIIMAN OLAVI ET AL) 3 September 1996 (1996-09-03)	1-4, 7-10,12, 13, 15-19, 22,23, 25,26,28
Y	abstract column 11, line 19 – column 18, line 3 ———	1-28
Y	DE 38 06 558 A (HITACHI LTD) 15 September 1988 (1988-09-15) abstract; claim 1 column 3, line 45 – line 56 ———	1-28
	-/--	

[X]	Further documents are listed in the continuation of box C.		[X]	Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
16 July 1999	06/08/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	FESTIN, R. ET AL.: "Detection of triple antibody-binding lymphocytes in standard single laser flow cytometry using colloidal gold, fluorescein and phycoerythrin as labels" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 101, 1987, pages 23-28, XP002109406 cited in the application	1-9,12, 15-22, 25,28
A	the whole document	10,11, 13,14, 23,24, 26,27

X	BÖHMER, R.M. ET AL.: "Immuno-Gold Labeling for Flow Cytometric Analysis" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 74, 1984, pages 49-57, XP002109407 cited in the application	1-9,12, 15-22, 25,28
A	the whole document	10,11, 13,14, 23,24, 26,27

T	FORTIN, MARYLENE ET AL: "Surface antigen detection with non-fluorescent, antibody-coated microbeads: an alternative method compatible with conventional fluorochrome-based labeling" CYTOMETRY, 36(1), 27-35 , 19 April 1999 (1999-04-19), XP002109408 the whole document -----	1-28

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